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Chemical Characterization and Biological Activity of Cork Sub-Products

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Mestrado em Química
Especialização em Química

Versão Definitiva
Versão Pública

Dissertação orientada por:
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2016

The presented thesis work was performed at the Chemical Biology and Toxicology (CBT) laboratory, incorporated into the *Research Institute for Medicines* (iMed.ULisboa) at the Faculty of Pharmacy, Universidade de Lisboa, under the supervision of Professor Isabel A. C. Ribeiro Monge da Silva (FFUL). The internal supervision was performed by Professor Susana Maria M. B. P. Pina dos Santos (FCUL).

Samples analysed in this thesis were kindly provided by SOFALCA, Lda.

"Eu queria, como tu, não saber que

os outros não valem nada

para os poder admirar como tu!

Eu queria que a vida fosse tão divinal

como tu a supões, como tu a vives!

Eu invejo-te, ó pedaço de cortiça

a boiar a tona d'água, à merce dos ventos,

sem nunca saber que fundo que é o Mar!"

Almada Negreiros

Agradecimentos

Em torno da realização deste projeto, nomeadamente no ambiente académico, gostava de agradecer:

À minha orientadora, Professora Doutora Isabel Ribeiro, que me acompanhou ao longo este projeto tão interessante e amplo e me acolheu nele com uma dedicação e paciência enormes, transmitindo sempre conhecimentos indispensáveis para a realização deste trabalho;

À Professora Doutora Susana Santos, que aceitou ser a orientadora interna, demonstrando sempre uma disponibilidade soberba;

À SOFALCA, por nos terem facultado as amostras e por nos receberem tão bem nas suas instalações, fornecendo informação privilegiada;

Ao Diretor da Faculdade de Ciências da Universidade de Lisboa, Professor Doutor José Martinho Simões, e à Diretora da Faculdade de Farmácia da Universidade de Lisboa, Professora Doutora Matilde Fonseca e Castro, por possibilitarem um acordo entre as Faculdades que me permitiu desenvolver este trabalho num ambiente novo;

Ao João Ferreira, responsável do equipamento de LC-MS pelo tempo investido nas análises necessárias durante este meu trajeto na FFUL;

Gostava de agradecer também aqueles que, fora do ambiente académico, me ajudaram a encontrar a motivação para continuar e lutar contra as adversidades, em especial:

À minha família, em especial aos meus pais e irmã, com os quais eu sempre pude contar nos bons e nos maus momentos, e que me ajudaram a ultrapassar as dificuldades ao longo deste ano;

À Sofia Assis, que me motivou e apoiou incomparavelmente;

Aos meus amigos, uns mais outros menos presentes, mas sempre dispostos a ajudar, e que levo no coração: Catarina Carapeta, Marisa Maia, André Janeiro, Paulo Coelho, Fábio Marques, Rúben Henriques, Patrícia Gírio e Diogo Pereira;

Ao *Dô* Clube de *Aikido*, por mais um ano de amizade, companheirismo e aprendizagem que tanto me molda física e espiritualmente, contribuindo para o meu desempenho académico;

Um muito obrigado!

Abstract

Cork is the outer bark of the Mediterranean tree *Quercus suber* L., and it is used in several industries due to aesthetic look, thermal and sound isolation capacities, although one of the main application is the production of cork stoppers. The cork, after being cleaned and grinded, is boiled and compressed (simultaneously), in order to agglutinate using the endogenous resin. Afterwards, the block is cut with the necessary dimensions. Cork sub-products (CKSP) are generated in the cork processing industry, and have a high content in organic compounds. Nowadays, environment-friendly behaviours are of extreme importance, so finding a way to reuse/recycle industrial residues is mandatory.

This work aims the chemical characterization and biological activity evaluation of cork sub-products in order to find possible applications for these sub-products, assuring a more sustainable cork production. CKSP were supplied by SOFALCA *Lda*, a cork processing company. Samples were characterized in terms of physical and chemical properties. The pH, the conductivity, the refractive index, the total dried residue, the turbidity and dissolved oxygen content of samples were assessed. In order to evaluate the chemical composition of samples, total phenolic content and total flavonoid content were determined using *Folin & Ciocalteu* and aluminium chloride methods, respectively. Both methods were performed with a microscale approach so a previous method validation was required. To identify the compounds present in the samples, Liquid Chromatography with *tandem* Mass Spectrometry (LC-MS/MS) was performed. Identification was based on LC-MS/MS spectrum, as well as multi reaction monitoring (MRM) approach with several standard compounds and their specific transition.

Antioxidant activity of the samples was assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, lipid peroxidation inhibition capacity assay and oxygen radical absorbance capacity (ORAC) assay.

Antimicrobial activity of samples was tested on planktonic cells by the disk diffusion assay and the broth microdilution method to assess the minimum inhibitory dilution, and on sessile cells using resazurin assay for biofilm quantification. Several microorganisms were used for the mentioned assays.

From the performed chemical and biological characterization, these sub-products revealed a great potential as antioxidant and antimicrobial complex mixture. Moreover, some sub-products revealed for the first time biofilm inhibition capacity that must be exhaustively studied in future work.

Keywords: *Quercus suber* L.; Cork sub-products; Cork sub-products constituents; antioxidant activity; antimicrobial activity.

Resumo

A cortiça é a casca obtida a partir da espécie *Quercus suber* L., uma árvore constituinte do ecossistema do *montado* vulgarmente conhecida como sobreiro, na qual funciona como casca. Frequentemente usada em diversas indústrias devido às suas capacidades isoladoras a nível térmico e acústico (como na construção civil e indústria automóvel), sendo uma das principais utilizações da cortiça a produção de rolhas. Esta matéria-prima é utilizada desde a antiguidade como vedante, tendo-se observado o aumento da sua importância económica a partir do século XX, quando se começou a utilizar a capacidade natural desta matéria em formar aglomerados. Hoje em dia cerca de 50 % da cortiça europeia tem origem portuguesa, representando uma importante fração da economia do país. Deste processo resultam vários tipos de subprodutos. Estes subprodutos apresentam um elevado conteúdo em compostos orgânicos que podem constituir um problema para o ambiente. Nos tempos atuais, estratégias e comportamentos amigos do ambiente são de extrema importância e, como tal, encontrar formas de reutilizar/reciclar resíduos industriais resultará numa mais-valia a nível económico e ambiental.

O presente trabalho tem como objectivo a caracterização química e biológica de subprodutos do processo de produção de derivados da cortiça, com vista a encontrar possíveis aplicações para estes resíduos, assegurando uma produção mais sustentável e amiga do ambiente. Para a realização deste trabalho foram necessárias amostras de subprodutos da indústria da cortiça, que foram cedidas pela Sociedade Central de Produtos de Cortiça, Lda. (SOFALCA).

Neste trabalho, foram realizados diversos ensaios em subprodutos da cortiça, com o intuito de caraterizar as propriedades físicas e químicas dos mesmos. Foi avaliado o pH, a condutividade, o índice de refração, o resíduo seco total, a turbidez e o oxigénio dissolvido. Sendo a cortiça um produto natural bastante rico em compostos fenólicos e flavonoides, foi analisado o conteúdo destes compostos nos subprodutos. O conteúdo em compostos fenólicos e em flavonoides foi avaliado pelo método de *Folin & Ciocalteu* e do cloreto de alumínio, respetivamente. Ambos os métodos foram realizados em microescala e, como tal, foi necessária uma validação prévia dos mesmos. Para a validação testou-se a linearidade, a gama de trabalho com base na homogeneidade de variâncias, o limite de deteção e o limite de quantificação e avaliou-se a repetibilidade do método. A avaliação do teor em compostos fenólicos e flavonóides foi obtida por comparação com a concentração em equivalentes de ácido gálhico e catequina, respetivamente. A identificação dos compostos presentes nos subprodutos foi feita recorrendo a cromatografia líquida associada a um sistema em *tandem* com um espectrómetro de massa do tipo triplo quadrupólo, com ionização por *electrospray*. A identificação dos compostos presentes nas amostras foi baseada nos espectros de LC-MS/MS obtidos e também recorrendo à abordagem de monitorização de reação múltipla, que utiliza padrões e as suas respetivas transições específicas.

A capacidade antioxidante dos subprodutos foi avaliada por métodos distintos, nomeadamente o método do 2,2-difenil-1-(2,4,6-trinitro)hidrazil (DPPH), o método de aferição da capacidade de inibição da peroxidação lipídica e da aferição da capacidade da absorver oxigénio radicalar (ORAC). Os subprodutos revelaram uma elevada capacidade antioxidante.

A actividade antimicrobiana dos subprodutos foi testada em células planctónicas usando o método da difusão de disco e o método da microdiluição para determinar a diluição mínima inibitória. Foi também estudada a capacidade de inibição no biofilme e quantificada a sua

viabilidade celular recorrendo ao método da resazurina. Foram utilizadas várias espécies de microrganismos para estes efeitos.

Em suma, os subprodutos estudados revelaram um grande potencial como fonte de compostos naturais com atividade antioxidante face a radicais livres. Apresentaram ainda propriedades antimicrobianas, com potencialidades mais específicas como a de diminuir a viabilidade do biofilme formado por algumas bactérias, ainda não reportado na literatura para amostras provenientes da indústria corticeira.

Palavras-Chave: *Quercus suber* L.*;* subprodutos da cortiça; constituintes de subprodutos da cortiça; atividade antioxidante; atividade antimicrobiana.

Publication of Results

Communications

Gomes, R., Santos, S., Duarte, N. e Ribeiro, I.A.C., "Implementation of microscale methods for quantification of phenolic and flavonoid compounds on natural products", 8th iMed. ULisboa Postgraduate Students Meeting (iPSC), 14 e 15 de Julho de **2016**, *Faculdade de Farmácia da Universidade de Lisboa*, Lisboa, Portugal.

Abbreviation and Symbols List

[M-H$^-$] – Deprotonated Molecule

AAPH - 2,2′-Azobis(2-methylpropionamidine) dihydrochloride

AO – Antioxidant

AUC – Area under the curve

BHI – Brain Heart Infusion

BHT – Butylated Hydroxytoluene

C – Catechin

C.I. – Confidence Interval

CA – Caffeic Acid

CE – Catechin Equivalents

CKSP – Cork Sub-Products

CLSI - Clinical and Laboratory Standards Institute

DAD – Diode Array Detector

DOC – Dissolved Oxygen Content

DPPH - 2,2-diphenyl-1-picrylhydrazyl

ECM – Extra Cellular Matrix

ESI – Electro Spray Ionization

FFUL – Faculdade de Farmácia da Universidade de Lisboa (Faculty of Pharmacy, University of Lisbon)

GA – Gallic Acid

GAE – Gallic Acid Equivalents

GPYA – Glucose-Peptone-Yeast extract Agar

HPLC – High Performance Liquid Chromatography

IC_{50} – Half maximum inhibitory concentration

ISO – International Standards Organization

J.C.G.M. – Joint Committee for Guides in Metrology

LC-ESI-MS/MS – Liquid Chromatography-Electrospray Ionization-Mass Spectrometry/Mass Spectrometry

LC-MS – Liquid Chromatography coupled to Mass Spectrometry

LOD – Limit of Detection

LOQ – Limit of Quantification

m/z – Mass-to-charge ratio

MHA – Muller Hinton Agar

MHB – Muller Hinton Broth

MID – Minimum Inhibitory Dilution

MMR – Multi Reaction Monitoring

MTP – Microtitier Plate

MTR – Methilicin Resistant

MTS – Methilicin Susceptible

N – Number of replicates

n – Refraction index

NATA – National Association Testing Authorities

NMR –Nuclear Magnetic Ressonance

OD – Optical Density

ORAC – Oxygen Radical Absorbance Capacity

ppb – parts per billion

ppm – parts per million

R^2 – Coefficient of determination

RI – Refractive Index

RNS – Reactive Nitrogen Species

ROS –Reactive Oxygen Species

RPMI - Roswell Park Memorial Institute

SD – Standard Deviation

TDR – Total dried residue

TE – Trolox Equivalents

TFC – Total Flavonoid Content

TIC – Total Ion Current

TPC – Total Phenolic Content

TSA – Trypto-caseín Soy Agar

TSB – Trypto-caseín Soy Broth

UV – Ultra violet

WHO – World Health Organization

λ – Wavelength

Index

Index of Figures

Index of Tables

Index of Annexes

Chapter 1 Introduction

"You must never be fearful of what you are doing when it is right."

Marie Skłodowska Curie

1.1 Discovering cork and it's applications

Cork is the bark of a Mediterranean tree, commonly found in Portugal, Spain, Morocco, Algeria, Tunisia and in some parts of France and Italy, named *Quercus suber* L. (Souza, 2012). These trees have high life-span, are able to sequester carbon dioxide from the atmosphere (Demertzi *et al.*, 2016) and can endure difficult weather conditions due to the protection made by the bark (Gil, 2015a). The EU is the world's biggest cork producer (80%), being almost 50% of the production originated from Portugal, playing a very important role in the economy and ecosystems of the country (Sierra-Pérez *et al.*, 2015).

There are three types of cork, with different economic and application interests. Producers have to wait about 20 to 30 years to remove the first produced cork, known as "virgin cork". After that, the cork-oak tree, by being exposed to several environmental agents, starts producing a new coating bark. This produced bark presents a traumatic nature due to recent exposition to environmental factors. After the removal of this second cork, the oak tree manages to produce a specific cork that reveals a homogeneous structure and growth, appropriate for the stoppers production. Both types of cork possess an inner tissue that differs from the virgin cork and conceals them different chemical composition (Silva, 2010).

Cork oak is extracted from the tree using cutting tools, where axes are usually the election tool, with a very cautious procedure, due to the risk of damaging the tree while cutting the oak, leading to death of the inner cells. That damage might interrupt the oak production around that spot, or in the worst case scenario, expose either the tree or the cutting material to parasites that have a host preference for *Quercus* trees, like *Biscogniauxia mediterranea*, commonly known as charcoal canker (Henriques, 2015) due to infected oak being similar to charcoal. *B. mediterranea* is known to be one of the higher impact factors on the wrecking of the *montado* ecosystem, with the capacity to kill cork-oak trees (Santos, 2003). Along with increased temperatures, wildfires and urbanization, these factors constitute serious problems to the cork production sector (Oliveira and Costa, 2012).

Nevertheless, cork is a widespread use material, and cork based products can be obtained through a sustainable removal (Silva, 2010). Cork is mainly used as a sealing agent since the ancient times, but only on the XX century a great commercial growth occurred (Gil, 2015a). It is so effective that champagne can be preserved in the original bottles for around 170 years. This was an occasional discover in 2010 when exploring a shipwreck on the Baltic Sea (Jeandet *et al.*, 2015). The shipwrecked champagne was chemically analysed and the results, compared to today's wines, allowed to conclude that besides having an extremely high sugar content (providing usual tastes for that time period) it revealed low levels of acetic acid, responsible for the specific taste of spoiled wines (Jeandet *et al.*, 2015).

Despite being used as stoppers, cork has many applications on nanotechnology, textiles (Nunes *et al.*, 2013), can be used as sorbent in different industries (*e.g.* pharmaceutical and wastewater treatment (Mestre *et al.*, 2007; Sfaksi *et al.*, 2014) and on the construction industry field. Cork can be used as a construction material due to the low thermal and electrical conductivity with low abrasion values that are independent from humidity and temperature. It is a flexible light weighted material capable of thermal, acoustic and vibration isolation (Gil, 2015b), and can be used as an aggregate for concrete (Matos *et al.*, 2015). This versatile material has been applied to new industries, like furniture, toys and automobile. In the last case, the luxury and decorative properties of cork are being used in gears and brake knobs, steering wheels and door panels for some brands prototypes, like Mercedes-Benz (Gil, 2015b). Still in the automobile industry, the weight and isolating properties of cork might make it a serious candidate to be used on renewable energies based cars (Gil, 2015b).

1.2 Chemical composition of Cork

In 1920, Klauber wrote the first report on cork composition, where he identified some of the main families of constituents. Yet, the first studies on the chemical composition of cork are dated from 1787 by Brugnatelli, to 1807 and 1815 by Chevreul, (Silva, 2010). Later on, Guillemont (1960) and Pereira (1988), amongst others, also presented extra composition results. Pereira studied the chemical composition of two different types of cork and was able to identify specific monosaccharides like glucose, xylose, arabinose, galactose, mannose and rhamnose. The results obtained by Klauber, Guillemonat, Pereira and Pinto regarding the chemical composition of cork are presented in *Table 1.1*.

From the chemical constitution of cell walls of cork, compounds are classified according to their structural and non-structural role. As structural compounds, suberin is a lipidic polimeric structure, responsible for the mechanical properties and the hydrophobic profile of cork (Fortes *et al.*, 2004). Also cellulose, one of the main structural polysaccharides, was identified as a structural constituent in 1927 by Zetsche and Rosenthal (Fortes *et al.*, 2004). Moreover, other polymeric macro molecules like lignin (obtained from cinnamyl alcohols (Markham, 1982) and polysaccharides are also part of the structural composition (Fortes *et al.*, 2004). Yet, the exact knowledge about the chemical structure of the main components of cork remains unknown (Fortes *et al.*, 2004).

Regarding the non-structural constituents, these are classified as extractives and inorganic. The first are low molecular mass compounds extractable using an adequate solvent with no alteration to the mechanical properties of the cell walls, while the inorganics are commonly known as ashes (Fortes *et al.*, 2004). Tannins and polyphenols can be obtained from the extractive fraction of cork (Gabrielli *et al.*, 2016; Gil, 2015a). The presence of elagitannins and phenolic compounds (e.g. vanillin, coniferaldehyde, coumarins, ellagic, gallic, protocatechuic and caffeic acids, and synaptic aldehyde) has been reported by some authors (Santos *et al.*, 2010), alongside with other several phenolic compounds and flavonoids (Conde *et al.*, 1998).

Nowadays, studies concerning the phenolic constituents of cork can be found on the literature (Santos *et al.*, 2013). Although the results vary and when comparing the reported composition results caution must be taken, due to the direct influence that the used method plays on the final outcome (Pereira, 2013), on top of the natural variability of cork from the same forest, (Pereira, 2013) and from the same tree (Conde *et al.*, 1998).

Studies towards the identification of phenolic extracts of cork allowing to identify compounds with molecular weight ranging 138 to 1176 g mol^{-1} have been reported. Some phenolic compounds, flavonoids and tannins are reported to be constituents of cork products (*Table 1.2*). Structures of some identified compounds are presented in *Figure 1.1* and *Figure 1.2*.

Over time, several of these phenolic and flavonoid compounds have been reported to present useful properties. An extensive research about the health benefits of natural phenolic and flavonoid compounds led to a high interest on these two families of compounds. Both families of compounds reveal radical scavenging, antioxidant and cytotoxic properties (Laranjinha e Cadenas, 1999). Moreover, their capacity to act as a natural protecting agent against microorganisms has been studied (Gil, 2015a).

Both natural hydroxybenzoic and hydroxycinnamic acids and their respective derivatives have antioxidant activity (Shahidi and Ambigaipalan, 2015). Hydroxybenzoic acids include gallic, protocatechuic (Brewer, 2011), ellagic (Salem *et al.*, 2016), vanillic, *p*-

hydroxybenzoic and syringic (Shahidi and Ambigaipalan, 2015), while hydroxycinnamic acids comprise of caffeic (Laranjinha and Cadenas, 1999), *p*-coumaric (Brewer, 2011), ferulic (Salem *et al*, 2016) and chlorogenic acids (Shahidi and Ambigaipalan, 2015). Flavonoids, specifically flavanones, like naringenin (Jabbari and Jabbari, 2016) and eriodictyol (Shahidi and Ambigaipalan, 2015) also reveal antioxidant capacity.

As antimicrobial agents, gallic and caffeic acids have been reported to inhibit growth of different types of bacteria such as *Escherichia coli*, *Staphyloccocus aureus* and *Pseudomonas aeruginosa* (Lima *et al.*, 2016). Also, *p*-hydroxybenzoic and protocatechuic acids were used to assess the growth inihibion of *Staphyloccocus aureus*, *Bacillus cereus*, *Escherichiea coli* and *Pseudomonas aeruginosa* (Gutiérrez-Larraínzar *et al.*, 2012), and ellagic acid inhibited *Vibrio chlorae*, *Shigella dysenteriae* and *Campylobacter spp* (Landete, 2011). In addition, ferulic and salicylic acids were able to significantly inhibit bacterial biofilm for *Bacillus cereus* and *Pseudomonas fluorescens* when used simultaneously (Lemos *et al.*, 2014).

Table 1.1 - Cork chemical composition according to different authors. [1]

Author	Cork type	Suberin (%)	Cellulose (%)	Polysaccharides (%)	Lignin (%)	Ashes/Others (%)	Ceroids (%)	Water (%)
Klauber, 1920	Non Specified	58	22	-	12	1	2	5
Guillemonat, 1960	Non Specified	45		12**	27	5	5	-
H. Pereira, 1988	Virgin	38.6	-	18.2	21.7	15.3	-	-
	Amadia	39.4	-	19.9	21.8	14.2	-	-
Pinto *et al*, 2009	Amadia	-		23*	33	4	-	-

* Carbohydrates

** Cellulose + Polysaccharides

[1] *Cited by Silva (2010).*

Table 1.2 - Identified phenolic compounds on cork related products over the last few years.

Compounds	Nº	Santos *et al*, 2010	Fernandes *et al*, 2011	Santos *et al*, 2013	Touatti *et al*, 2015
Quinic acid	(1)	✓		✓	
Gallic acid	(2)	✓	✓	✓	✓
p-hydroxyphenyllactic acid	(3)	✓		✓	
p-hydroxybenzoic acid	(4)	✓			
Esculetin	(5)	✓		✓	
Caffeic acid	(6)	✓	✓	✓	✓
Vanillin	(7)	✓	✓	✓	✓
Vanillic acid	(8)	✓			✓
p-coumaric acid	(9)	✓		✓	
Ferulic acid	(10)	✓	✓	✓	
Ellagic acid	(11)	✓	✓	✓	✓
Salicylic acid	(12)	✓			
Eriodictyol	(13)	✓		✓	
Naringenin	(14)	✓			
Protocatechuic acid	(15)	✓	✓	✓	✓
Protocatechuic aldehyde	(16)		✓		✓
Coniferaldehyde	(17)		✓	✓	
Valoneic acid	(18)		✓		
Methyl gallate	(19)			✓	
Isorhamnetin	(20)			✓	
Castalagin/Vescalagin	(21)		✓		✓
Syringaldehyde	(22)				
Ellagic acid pentose	(23)		✓	✓	✓
Ellagic acid deoxyhexose	(24)		✓		
Ellagic acid hexose	(25)		✓		
Valoneic acid dilactone	(26)		✓	✓	✓
HHDP-glucose	(27)		✓		✓
Dehydrated tergallic-C-glucoside	(28)		✓		
HHDP-galloyl-glucose	(29)		✓		
Trigalloyl-glucose	(30)		✓		
Di-HHDP-glucose	(31)		✓		
HHDP-digalloyl-glucose	(32)		✓		
Di-HHDP-galloyl-glucose	(33)		✓		
Trigalloyl-HHDP-glucose	(34)		✓		
Pentagalloyl-glucose	(35)		✓		
Mongolican A/B	(36)		✓		
Brevifolin-carboxilic acid	(37)			✓	✓
Caffeic acid isoprenyl ester	(38)			✓	✓
Ellagic acid rhamnoside	(39)			✓	✓
Isorhamntine-rhamnoside	(40)			✓	✓
Chlorogenic acid	(41)				✓

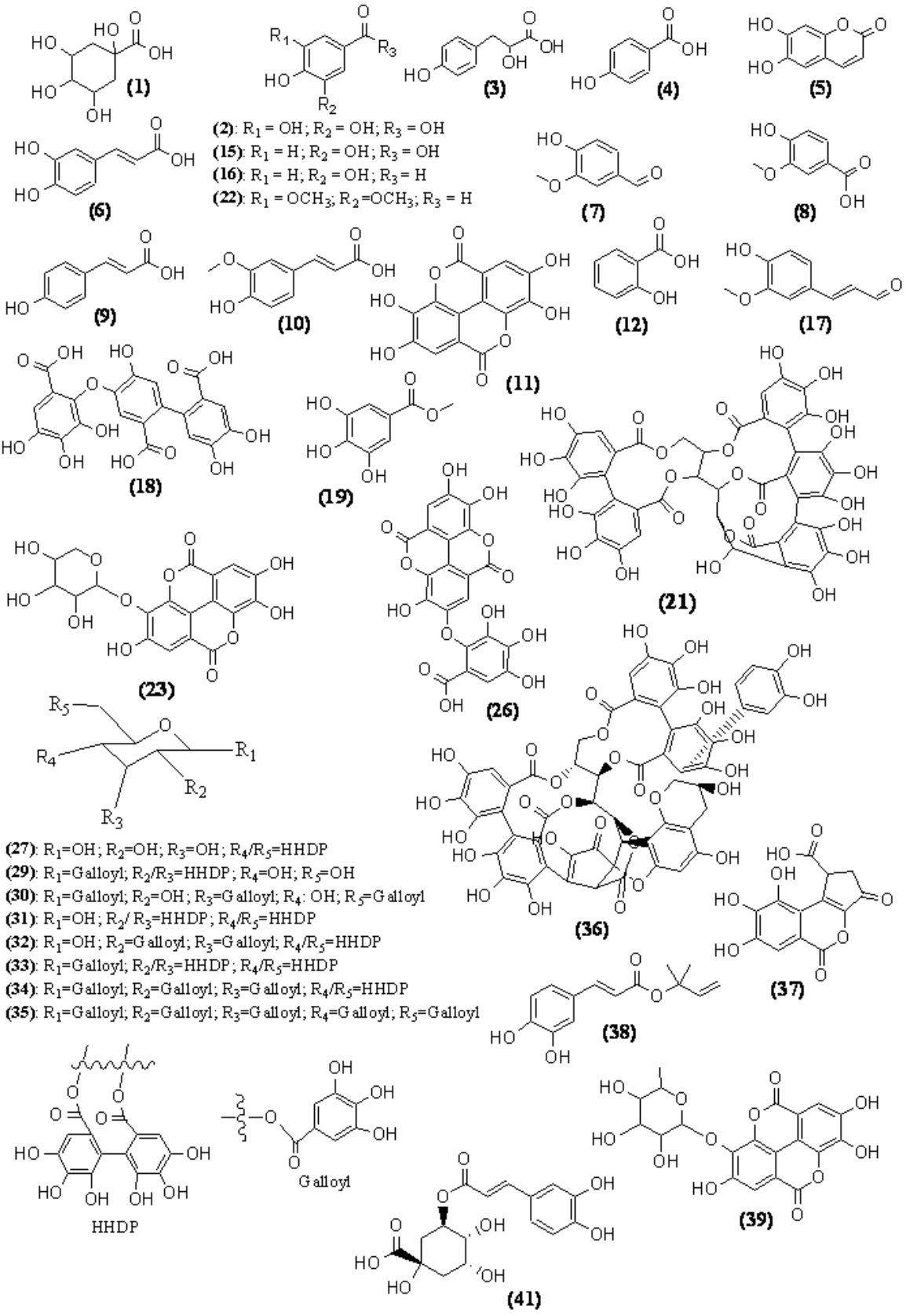

Figure 1.1 - Chemical structures of some of the identified phenolic compounds present in cork.

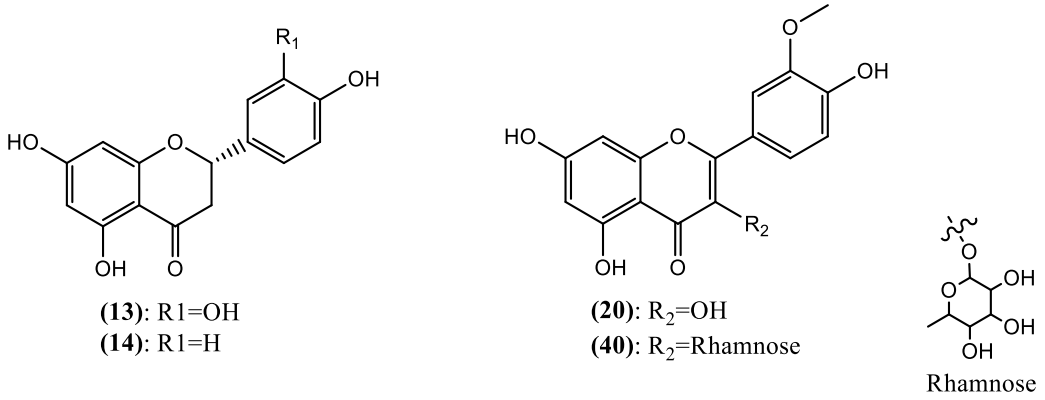

Figure 1.2 - Flavonoids identified as cork constituents.

AIM

This thesis aims to study subproducts resulting from the transformation of cork by SOFALCA, in an attempt to find a potential application for this kind of CKSP. The studies will focus on the samples constituents, their antioxidant properties, and on their antimicrobial properties towards several microorganisms.

Thesis outline

The thesis is organized in 5 chapters that cover the research aims previously mentioned. The introduction is presented in Chapter 1, the final remarks (alongside some future perspectives using this kind of samples) on Chapter 5 and the addressed research areas are organized as follows.

In Chapter 2, physical and chemical analysis are reported. CKSP were characterized in terms of pH, conductivity, refraction index, total solid residue, turbidity and dissolved oxygen. The phenolic and flavonoid contents were determined, and LC-MS was used to identify some of the present compounds.

In Chapter 3, the results of the antioxidant properties of cork sub-products can be found. The antioxidant properties were determined towards DPPH (mimicking RNS) and peroxyl radicals, known as endogenous ROS.

In Chapter 4, the antimicrobial properties of the samples were assessed, using several microorganisms strains, including also the ability to inhibit biofilm formation.

Chapter 2 Physical and chemical characterisation of CKSP

"*All that is gold does not glitter,*
Not all those who wander are lost;
The old that is strong does not wither,
Deep roots are not reached by the frost"

J.R.R. Tolkien

2.1 Introduction

CKSP are reported to be rich in phenolic compounds, where phenolic acids are the greater part (Santos *et al.*, 2010). The presence of phenolic compounds like gallic acid, *p*-hydroxyphenyllactic acid and vanillin, amongst others, was reported in the literature (Santos et al., 2010).

Measuring pH using accurate and reliable methods in chemical, biological, clinical, industrial and environmental samples is of great importance (Ertürün *et al.*, 2015). Based on the presence of certain compounds and their acid properties, the acidity of the medium varies and the pH of the CKSP is related to their components profile.

Another property that is related to the sample composition is the sample conductivity. The ability of a liquid sample to convey electricity is the specific conductance, also known as electrical conductivity. This ability is influenced by the presence of ionic compounds (Uwidia and Ukulu, 2013), and increases with the amount of ions in the sample.

CKSP refractive index can also be evaluated. Refractive index is a property intrinsic to all media, commonly used to evaluate the purity of the liquid since it is related with the density. It can be used either for quality control purposes, on food or cosmetic industries, or for environmental control (Räty and Peiponen, 2015).

Liquid Chromatography coupled to mass spectrometry (LC-MS) is one of the most used methodologies to identify a sample composition. LC-MS is used to identify some of the organic compounds present in natural products extracts, like CKSP (Conde *et al.*, 1998; Fernandes *et al.*, 2011), allowing identification of phenolic compounds, flavonoids and tannins as the main extractive compounds. Some phenolic compounds already reported to be cork constituents, like caffeic acid, are used in pharmaceutical, chemical and food industries (Daâssi *et al.*, 2014).

On this part of the work, the available CKSP samples were submitted to several physical and chemical characterization assays such as evaluation of pH, conductivity, refraction index, total solids residue and dissolved oxygen content. The composition on phenolic and flavonoid compounds was also assessed using *Folin & Ciocalteu* and aluminium chloride methods and LC-MS/MS.

2.2 Material and reagents

The following reagents were used: aluminium chloride hydrate, *Folin & Ciocalteu's* reagent, sodium carbonate and sodium thiosulphate padronized solution (1 mol L^{-1}) purchased from Sigma Aldrich (Steinheim; Germany), manganese sulphate purchased from May and Baker chemicals (London, England); sodium azide adquired from Carlo Erba (Barcelona, Spain); conductivity sodium chloride standard solutions (1.413 mS cm^{-1} and 12.88 mS cm^{-1},) obtained from Crisom (Barcelona, Spain); pH standard solutions (pH values 4 and 7) from Metrohm (Herisou, Switzerland); sodium hydroxide and sodium nitrite obtained from V. Reis, Lda (Lisboa, Portugal); starch, methanol absolute, sulphuric acid and acetonitrile HPLC grade were obtained from Merck (Darmstad, Germany); formic acid purchased from AnalaR (Pool, England). The LC-MS standards (quinic acid, gallic acid, protocatechuic acid, *p*hydroxybenzoic acid, caffeic acid, vanillin, *p*-coumaric acid and ellagic acid) were obtained from Extrasynthese (Genay, France).

2.2.1 Equipment

The following equipment was used: *Metrohm 744 pH Meter* using a Metrohm electrode (Precision ± 0.01); *P Selecta Ultrasons bath; Hitachi UV 2000* spectrophotometer (Precision ± 10^{-3} A.U.); *Wayne Kerr B905* automatic precision bridge with an Ingold conductivity cell type 980-k19/120 (platinum electrodes), (Precision ± 0.0001 mS); *Bellingham + Stanley Abbe 5* refractometer (Precision ± 0.01); *Heraeus* drying oven; *METTLER AE 200* balance (Precision ± 0.0001 g); *SPECTROstar Omega* micro plate absorbance reader (Precision ± 0.001), controlled by *Omega* software; *Eutech Instruments TN-100 Turbidimeter*.

2.2.2 Samples

Samples were collected from different points of cork production and for three distinct harvests.

2.3 Methods

2.3.1 pH measurement

The pH parameter is related to the concentration of $[H^+]$ in solution, where higher concentrations are numerically expressed as lower pH values, expressing the acidity of a solution. Before each set of pH measurements, electrode calibration was performed, using two standards solutions, with pH 4 and 7.

2.3.2 Conductivity

Conductance values were obtained using a Wayne-Kerr B905 automatic precision bridge (WKR, England) using an Ingold conductivity cell type 980-K19/20 with platinium electrodes and a cell constant of 1.004 cm^{-1}, operating at 25 ºC and 1 kHz. Two Crison KCl standard solution were used to determine the cell constant. To obtain the samples conductivity, *Equation 2.1* was used:

$$\boldsymbol{Conductivity = conductance \times cell's\ constant} \qquad \textit{Equation 2.1}$$

2.3.3 Refraction index

Refraction index (RI) stands for the path described by light in a medium and corresponds to the phase velocity of light in a medium divided by its velocity in vacuum (Räty and Peiponen, 2015). Therefore, such a parameter depends not only on the refractive index of the medium, but also on the molecular structure, the wavelength of the light, temperature and the pressure of the medium (Räty and Peiponen, 2015).

For the refraction index determination of CKSP, 3-5 drops of sample were placed on the lower prism assuring that all the surface was covered and no air bubbles were observed before both prisms were joined together. MilliQ deionized water was used as assay control. Assays were performed at 25 ºC using direct sunlight.

2.3.4 Turbidity

Turbidity is defined as the reduction of transparency of a medium (usually liquid) caused by the presence of undissolved matter (usually small particles), causing light to scatter (Lawler, 2005). This property can be evaluated by nephlometry, measuring directly the light scattering degree of the medium. It is a non-destructive method that allows the quantification of particles in a media.

Nephlometry was the selected method to evaluate turbidity of filtered and non-filtered CKSP. Before each set of measurements, a set of 4 standards (*i.e.* 0.02; 20; 100 and 800 NTU) were used to calibrate the equipment. Each sample was measured in triplicate and final results were presented as the mean ± SD.

2.3.5 Total dried residue

In liquid samples, the word "solid" is related to suspended or dissolved compounds, able to be physically isolated, after free water evaporation. For determination of the total dried residue (*i.e.* suspended and dissolved solids) the gravimetric method, according to Hach (2015), was used.

For the total dried residue (TDR), the samples were placed in a porcelain evaporating dish and were left inside a drying oven, with temperature set for 105 ºC (*Figure 2.1*) until the mass variation of the residue was equal or smaller than 4 mg. Before being weighed, the samples were cooled down inside a desiccator.

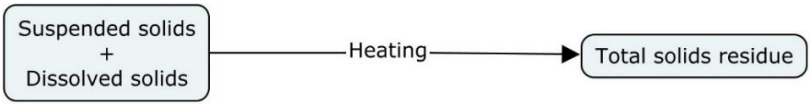

Figure 2.1 - Scheme of total solid residue procedure and its respective samples.

2.3.6 Dissolved Oxygen Content

Dissolved oxygen Content (DOC) was performed according to **ISO standards (1983)**. The method is based on the reaction of dissolved oxygen with manganese (II) hydroxide.

Dissolved oxygen was achieved by the iodide equivalents, obtained by titration and subtracted of other reducing agents present in the solution.

i) Presence of oxidant/reducing agents

First, it was necessary to find if the samples presented oxidant or reducing agents. For that, two different procedures were performed:

Presence of oxidant agents

CKSP were added of 2 drops of phenolphthalein (1 g L^{-1} of an ethanolic solution), followed by 0.5 mL of concentrated sulphuric acid and approximately 0.5 g of iodide potassium. A few drops of starch indicator (10 g L^{-1}) were added and. if the solution turned blue oxidizing substances were present (Solution A).

Presence of reducing agents

To the obtained solution (Solution A), 0.2 mL of iodine solution (0.005 mol L^{-1}) were added. If the solution remained colourless, reducing agents were present.

ii) Oxygen and other reducing agents quantification

To a 50 mL sample aliquot, 1 mL of manganese sulphate 45 % (w/v) was added, followed by 1 mL of an alkali solution (sodium hydroxide 0.35 %; potassium iodide 30 %; and sodium azide 0.01 %) (w/v). The mixture was shaken and 1 mL of concentrated sulfuric acid was added. A few drops of starch (10 g L^{-1}) were added, and titrated with a standard sodium thiosulphate solution (0.25 mol L^{-1}).

In the presence of other reducing agents, a correction to the first titration was needed. In that way, to 50 mL of sample, 1.5 mL of concentrated sulphuric acid were added, followed by 2 mL of alkali solution, and 1 mL of manganese sulphate 45%. A few drops of starch solution were used as indicator, and it was titrated with a 4 g free chloride L^{-1} solution.

This second titration was used as a correction factor due to the presence of reducing substances that affect the true result obtained on the first titration. A correction was made, according to *Equation 2.2* and *Equation 2.3*:

$$O_2\left(\frac{mg}{L}\right) = \frac{MM\ (O_2) \times V_{tit} \times C_{tit} \times f_1}{4 \times Volume_{sample\ for\ 1st\ titration}} - \frac{MM\ (O_2) \times Volume_{tit\ 2nd\ titration} \times C_{tit}}{4 \times Volume_{sample\ for\ 2nd\ titration}}$$

Equation 2.2

$$f_1 = \frac{Volume_{\ sample\ for\ 1st\ titration}}{Volume_{\ sample\ for\ 1st\ titration} - (Volume_{\ Manganese\ sulphate} + Volume_{\ Alkali\ reagent})}$$

Equation 2.3

Where $MM(O_2)$ is the molar mass of oxygen; V_{tit} is the volume used on the titration; C_{tit} is the concentration of the titrant, $Volume_{\ sample\ for\ 1st\ titration}$ is the volume of sample used for the first titration (quantification), $Volume_{\ sample\ for\ 2nd\ titration}$ is the volume of sample used for the second titration (correction), $Volume_{\ Manganese\ Sulphate}$ is the volume of manganese sulphate used for the respective titration, $Volume_{\ Alkali\ Reagent}$ is the volume of alkali reagent used for the respective titration.

2.3.7 Total Phenols and Flavonoids Quantification

The methods used for determination of total phenolic content (TPC) and total flavonoid content (TFC) have been described on the literature, however, when performing a large amount of samples, those can become time consuming and require high reagent needs. Therefore, in the present work, a microscale approach was adopted to quantify TPC and TFC. To assure method suitability a short method validation procedure was followed according to **ISO standards (1990; 1993)**.

2.3.7.1 Micro methods validation

For the validation of these two methods, some important aspects were evaluated to assure the method suitability such as linearity, working range, limit of detection (LOD), limit of quantification (LOQ), and repeatability.

2.3.7.1.1 Linearity

Linearity is a calibration characteristic, defined as the ability to induce a response that is directly proportional to the given analytical parameter (NATA, 2013). To obtain the calibration curve, a linear regression was applied to the obtained data. After obtaining the residuals standard deviation to the data using a linear ($y = mx + b$) and a quadratic regression ($y = ax^2 + bx + c$), the standard deviation of both the linear and the quadratic regressions, a *F*-test was used to assess the best model between the two previously mentioned regression, according to *Equation 2.4* and *Equation 2.5*:

$$\boldsymbol{DS^2 = (N-2) \times s^2_{\frac{y}{x}} - (N-3) \times s^2_{y^2}} \qquad \textit{Equation 2.4}$$

$$\boldsymbol{PG = \frac{DS^2}{s^2_{y^2}}} \qquad \textit{Equation 2.5}$$

Where $s^2_{y/x}$ stands for the residual variance when a linear regression is applied and $s^2_{y^2}$ for the residual variance when a quadratic regression is applied, being *N* the number of concentration levels, which in this case, according to ISO Standards (1990), can be *N=10*. After obtaining the result of the test value (*PG*), it is compared with the tabled *F* value, for each specific number of degrees of freedom. If the test value is greater than the *F* value, it is possible to say that the linear regression does lead to a worse adjustment. On the order hand, if the test value is smaller than the *F* value, non-linear regression does not change the adjustment of the values in a significant way, and linear function can be accepted as the best function to describe the data on such a working interval.

Moreover the linearity assessment was also performed by evaluating the determination coefficient (R^2) and by plotting the residuals against the concentration (logarithm of concentration). If a deviation lower than 15 % is observed then the results are within the acceptable interval.

The residues were obtained according to *Equation 2.6*:

$$Normalized\ residue\ (\%) = \frac{Conc._i}{Abs._i} \times \frac{Conc._n}{Abs._n} \times 100 \qquad \textit{Equation 2.6}$$

Where $Conc._i$ and $Abs._i$ are the concentration and the absorbance of the *i* replicate, respectively; $Conc._n$ and $Abs._n$ are the concentration and the absorbance of the point in the middle of the calibration curve, respectively.

2.3.7.1.2 Working range

The working range is known to be the interval of a certain property, inherent to the method, where the most expected value of the analysis should be as near to the centre as possible, allowing determination through the best suited function. The working range can also be defined as the concentration range where the method can be used. The working range suitability is obtained by the homogeneity of variances.

The homogeneity of variances test, for the lower and upper range limits, using a total of 10 replicates per each, was evaluated. The variance is evaluated with an *F*-test. After obtaining the necessary data, the homogeneity of variances was assessed with the edges of the calibration curve. First, the mean of the replicates of each edge was calculated using *Equation 2.7*, and then the standard deviation was obtained according to *Equation 2.8*.

$$\bar{y}_\iota = \frac{\sum_{j=1}^{10} y_{i,j}}{n_i} \; ; i = 1 \; or \; i = 10 \qquad \textit{Equation 2.7}$$

$$s_i^2 = \frac{\sum_{j=1}^{10} (y_{i,j} - \bar{y}_\iota)^2}{n_i - 1} \qquad \textit{Equation 2.8}$$

After obtaining both values, where s_1^2 stands for the standard deviation of the first standard solution and s_{10}^2 for the standard deviation of the last standard solution, the test value is obtained by the division of the higher by the smaller value, *i.e.*, using the formulas presented in *Equation 2.9* and *Equation 2.10*.

$$PG = \frac{s_1^2}{s_{10}^2} \qquad \textit{if } s_{10}^2 \textit{ is smaller than } s_1^2 \qquad \textit{Equation 2.9}$$

$$PG = \frac{s_{10}^2}{s_1^2} \qquad \textit{if } s_1^2 \textit{ is smaller than } s_{10}^2 \qquad \textit{Equation 2.10}$$

Once the test value is calculated, it is possible to make decisions about the working range of the method. For instance, if the test value is smaller than the *F* tabled value, it means that the difference on variance between these two edges is not significant, and that work range

can be used. If not, adjustments on the work range should be made until the value of *F* tabled is greater than the test value.

2.3.7.1.3 LOD/LOQ

Two important parameters that must be determined are the Limit of Detection (*LOD*) and Limit of Quantification (*LOQ*). The first is described as the smallest amount of an analyte that can be reliably detected by a given method (J.C.G.M., 2008). The second, *LOQ*, is the minimum amount of analyte that can be quantified.

LOD and *LOQ* can both be calculated using the residual standard deviation of the linear regression and the slope of the previous one, according to *Equation 2.11* and *Equation 2.12.*

$$Y(LOD) = \frac{3,3 \times s_{y/x}}{b} \qquad \textit{Equation 2.11}$$

$$Y(LOQ) = \frac{10 \times s_{y/x}}{b} \qquad \textit{Equation 2.12}$$

2.3.7.1.4 Repeatability

Repeatability is defined as the precision obtained from the measurements performed in repeatability conditions, *i.e.*, same operator, same equipment, lab conditions, in a short period of time, and it can be expressed in different ways (Bettencourt, 2014). In these methods, the repeatability was assessed as the standard deviation of ten standard solutions (*N*=10) of gallic acid with a concentration of 2 and 10 ppm for the TPC assay and ten standard solutions of catechin with a concentration of 4 and 20 ppm for TFC assay.

2.3.2 Total Phenolic content (TPC)

TPC was obtained from *Folin & Ciocalteu's* method, based on the reaction of *Folin & Ciocalteu* reagent with the phenols present in the sample, allowing colour to change from strong yellow to pale blue. The quantification was performed by measuring the samples absorbance and comparing to a calibration curve, as reported by Singleton *et al.* (1999) with few adjustments to the microscale approach.

For the calibration curve, a 100 ppm stock solution (II) of gallic acid (GA) was prepared from a previous stock solution (I) of GA 1000 ppm, both prepared on methanol/water [8:2] mixture. From the final stock solution (II), standard aqueous solutions with concentration ranging from 2 to 10 ppm were prepared.

From each standard solution, 262.5 µL were transferred to a 96-well microtiter plate (MTP), and 7.0 µL of *Folin & Ciocalteu*'s reagent was added. After 3 min, each well was supplemented with 30 µL of sodium carbonate aqueous solution (35 %). The MTP was kept under dark and at room temperature for 1 h, and the absorbance at λ= 725 nm was measured on a *SPECTROstar Omega* micro plate absorbance reader.

Samples were processed and diluted to fit the calibration curve (2-10 ppm of *GAE*), working range. Measurements were performed in triplicates, water was used as blank, and the final results were expressed as the mean ± SD of GA equivalents (ppb/GAE).

TPC was determined according to a linear function obtained from the calibration curve, *i.e.*, $x = \frac{y-b}{m}$,

where *x* is the concentration of the sample, *y* is the absorbance value of the sample, *b* is the intercept value, and *m* is the slope.

2.3.3 Total Flavonoid Content (TFC)

TFC determination was performed according to the aluminium chloride method reported by Zhishen *et al.* (1999) with few adjustments. The method is based on the formation of stable aluminium-flavonoid complexes (Bag *et al.*, 2015), resulting from the reaction of aluminium chloride with the flavonoids that originates a colour change from pale yellow to a strong pink.

Final complex absorbance at λ= 510 nm was measured and TFC was determined using a calibration curve, *i.e.*, $x = \frac{y-b}{m}$,

where *x* is the concentration of the sample, *y* is the absorbance value of the sample, *b* is the intercept value, and *m* is the slope.

For the TFC quantification, a stock solution (SS) of catechin (C) at 1000 ppm was prepared and standard solutions, with concentration ranging 2-20 ppm, were obtained by dilution of SS. From each standard solution, 150 µL were transferred to a 96-well microtiter plate and 36 µL of an aqueous solution of sodium nitrite (5 %) (w/v) were added. After 5 min, each well was supplemented with 36 µL of an aqueous solution of aluminium chloride (2.5 %) (w/v) and, after 6 min, with 132 µL of an aqueous solution of sodium hydroxide (0.8 mol L^{-1}). Absorbance was measured at λ= 510 nm on a *SPECTROstar Omega* microtiter absorbance reader.

The samples were processed to fit the calibration curve (2-20 ppm of *C*), quantification was performed in triplicates, water was used as blank, and the final results are reported as the mean ± SD of Catechin Equivalents (ppb/CE).

2.3.4 Characterization of samples content by LC-MS/MS

LC-MS is a combination of two distinct techniques that brought new perspectives when conceived. It coupled the liquid chromatography to the mass spectrometry, complementing other hyphenised techniques, by being able to analyse non-volatile and thermolabile compounds. Moreover, one of the most important benefits was the fact that aqueous matrix could be analysed with no previous extraction or derivatization, what led, mostly, to economic benefits (Nogueira, 2014).

The used equipment, commonly known as *Tandem*, consists on an High Performance Liquid Chromatography (HPLC) with a diode array detector (DAD) coupled to a MS/MS analyser, allowing to obtain some structural information, since the selected *m/z* can be fragmented again. In this case, electro spray ionization (ESI) was the ion source and the used mass analyser was a triple quadrupole.

For the LC-ESI-MS/MS analysis, a *RP18 LiChro CART 250-4* (250 mm, 4 mm i.d.; 5 µm) column from Merck was used, in a *Waters™ Alliance 2695 HPLC Separation Module* coupled to a *Waters™ Micromass Quatromicro API Tandem Quadrupole Mass Spectrometer* equipped with a *Waters™ electrospray ionisation source* (ESI), controlled by *Waters™ Empower Software*. The method was optimized for negative ESI mode with 135 min runs, and a column temperature of 40 ºC. Mobile phase consisted of water: formic acid (95.5 %:0.5 %) (eluent A) and acetonitrile (eluent B). The following elution programme was used: isocratic for 0.1 min at 100% of eluent A; gradient for 70 min until 31.5% of eluent B; gradient for 30 min until 90% of eluent B; isocratic for 10 min with 90% of eluent B; gradient for 5 min until 100% of eluent A and isocratic for 15 min with 100% of eluent A.

Samples were kept at 4 ºC, injection volume was 20 µL and the used flow rate was 0.3 ml min^{-1}. ESI capillary voltage was 3.00 kV, with a cone voltage set on 30 V and cone gas flow of 50 L per hour. Before being injected, samples were filtrated using a *Schleider & Schuell* paper filter nº595, and again using a 0.45 µm polypropylene syringe filter.

Chromatograms were obtained at λ= 254 nm and λ= 280 nm. Full mass scan MS data over the *m/z* range 100-1000 were obtained in order to have information about the molecular ion of each compound separated. To study the fragmentation pattern of the compounds present in samples, MS/MS experiments were performed using ionization energy of -20 keV and collision energies ranging from 0.5 to 50. Samples were also analysed using Multiple Reaction Monitoring (*MRM*) by comparing to a mixture of standards containing quinic, gallic, protocatechuic, *p*-hydroyphenyllactic, caffeic, *p*-coumaric and ellagic acids, and vanillin. Each compound on the standards mixture was fragmented using their specific transitions, as shown in *Table 2.1*, and compared to samples.

Table 2.1 - MRM specific transition information for the used standard compounds.

Compound	Information on specific transition
Quinic acid	191>85
Gallic acid	169>125
Protocatechuic acid	153>109
***p*-hydroxyphenyllactic acid**	137>93
Caffeic acid	179>135
Vanillin	151>135
***p*-coumaric acid**	163>119
Ellagic acid	301>284

2.4. Results

2.4.1 Physical and chemical characterisation

After receiving the CKSP from SOFALCA, some physical and chemical characteristics were determined, like pH, conductivity, RI, TDR, turbidity and DOC, as a first approach to characterize those samples. All samples were analysed.

2.4.2 Validation

Neither *Folin & Ciocalteu* for determining TPC nor aluminium chloride method for determining TFC had been applied on the lab by a micro method approach, so validation of these two methods was necessary. Validation was then performed, according to **ISO 8466-1 1990** and **ISO 8466-2 1993**, as mentioned in section *2.3.7.1*.

2.4.2.1 TPC and TFC method validation

For the TPC method validation a working range of 2 to 10 ppm of gallic acid was chosen. The working range was first tested for 1 to 10 ppm, but the differences of the variances were significant, so the working interval had to be adjusted (*i.e.* 2 to 10 ppm). For the TFC the selected working ranged from 4 to 20 ppm of Catechin. Calibration curves were obtained from plotting the absorbance *vs* the concentration of the standards used for each method, on the working range. Linearity equations were obtained using a linear regression analysis given by *GraphPad*, obtaining $y = 0.0755x - 0.0024$ with a R^2 of 0.9960 and $y = 0.0141x - 0.0013$, with R^2 of 0.9955 for TPC and TFC, respectively (*Figure 2.2)*. The results for the method validation parameters for TPC and TFC are presented in *Table 2.2*.

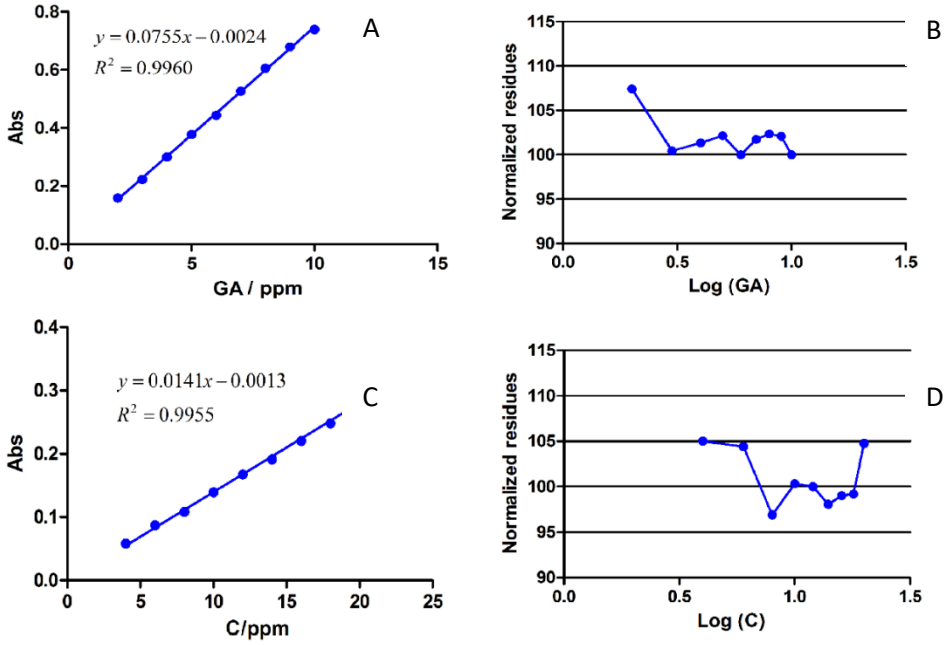

Figure 2.2 - TPC and TFC obtained calibration curves and normalized residues. A – *TPC calibration curve*; B – *TPC normalized residues*; C – *TFC calibration curve*; D – *TFC normalized residues*.

Table 2.2 - Linearity validation parameters for TPC and TFC.

Method Validation Parameters (99.5 % C.I.)	**TPC**	**TFC**
Working interval	2-10 ppm /GA	4-20 /C
R^2	0.9960	0.9955
Deviation of the normalized residues	< 15 %	< 15 %
$s^2_{y/x}$	3.67×10^{-5}	3.05×10^{-5}
$s^2_{y^2}$	0.012	0.028
DS^2	-0.083	-0.198
PG (linearity)	-6.97	-6.99
F(crit)	4.15	3.68
LOD	0.269	1.296
LOQ	0.816	3.927
Homogeneity of variances		
s^2_1	2.00×10^{-5}	7.8×10^{-6}
s^2_{10}	8.86×10^{-5}	2.5×10^{-5}
PG(variances)	4.41	3.23
F(crit)	5.47	5.35
Repeatability (RSD %)		
s_1	2.70	5.27
s_{10}	1.22	2.50

From the validation results presented in *Table 2.2* it is possible to conclude that the chosen working range for TPC determination (2-10 ppm of GA) and for TFC determination (4-20 ppm of C) was suited for the linear regression model. *F*-test results reveal that there are not any significant differences on the variances of the lower and upper limits, on a 95 % Confidence Interval (CI), according to **ISO standards (1990)**. LOQ for TPC and TFC were 0.816 ppm and 3.92 ppm respectively and were both inferior than the lower limit of the calibration curve, meaning that quantification is possible on the working range. As for repeatability, it was determined for 2 concentration levels of both GAE and CE, one on the lower limit and another on the upper limit, obtaining a relative standard deviation of 2.70 % and 1.22 % for TPC and 5.27 % and 2.50 % for TFC, respectively. With the reported conditions, the methods were suitable for the quantification of TPC and TFC.

2.4.3 TPC and TFC content

It is known that raw cork has extractable compounds where phenolic and flavonoids compounds are included (Gil, 2015a). These phenolic compounds have been reported to be active against microorganisms (Gil, 2015a), so determination of the sample content on those compounds is of great interest.

2.4.4 LC-MS results

As a first approach to LC-MS, a 130 min chromatography run was made. A total ion current (TIC) chromatogram was obtained revealing the peaks of possible isolated compounds. For each peak, full scan MS data over the *m/z* range of 100-1500 were obtained to reach information about the corresponding deprotonated molecule and further conduct MS/MS experiments.

2.5. Conclusion

CKSP samples were supplied by SOFALCA (Abrantes, Portugal), and sampling was performed in three different harvestings, allowing to study the CKSP composition on different seasons.

From the obtained physical and chemical characterization results CKSP can be a risk to the environment. Such a fact urges even more the need to find new applications for the CKSP studied through this work.

After quantification of TPC and TFC, it is possible to conclude that samples showed a high content of each family of compounds commonly present on natural products, and that might play an important role on biologic environments, due to their anti-inflammatory, anti-bacteriological and, specially, their antioxidant activity. Since antioxidant activity can be an important and a valuable application for the compounds in these samples, it would be of great interest to study it, since these compounds can be applied on different areas.

Using LC-MS, identification of phenolic compounds was achieved, by comparing with the results obtained by other authors and using MRM with a mixture of standards. Several phenolic compounds were identified.

Chapter 3 Antioxidant activity

“If.

'If' always propelled my thoughts back to the present, because 'if' depended so much on keeping my wits about me. I couldn't properly sense things if I was distracted. 'If' demanded my full presence and participation in 'now.

'If', as much as it scared me, also kept me sane.”

Ransom Riggs

3.1 Introduction

Due to the chemical composition of the cork from *Quercus suber* L. on phenolic and flavonoid compounds and based on the properties presented by these families of compounds that include antioxidant and radical scavenger capacity, that are not interdependent (Laranjinha and Cadenas, 1999), studies towards these kind of characterizations have been performed on the last few years (Santos *et al.*, 2010; Santos *et al.*, 2013; Touati *et al.*, 2015).

Oxidative stress is a known disproportion between reactive oxygen species (ROS)/ reactive nitrogen species (RNS) and antioxidants (Biosciences), usually on physiological environment (Macdonald-Wicks *et al.*, 2006). On normal physiological conditions, those ROS are neutralized by the antioxidant enzymes of the cell and other redox compounds that the cell might have. ROS are important to neutralize and it is not always possible to do so fast enough to avoid some cellular damage. Although there is not an official international definition of "antioxidant", this refers to a compound that is able to prevent or delay oxidation of easily oxidisable material (Macdonald-Wicks *et al.*, 2006).

Phenolic compounds and flavonoids are known to act as antioxidants due to their ability to stop the chain-reaction oxidations, since they have high susceptibility to react (Mann *et al.*, 1994). This susceptibility, allied to their anti-inflammatory and antimicrobial properties (Santos *et al.*, 2010), makes them important to human health, as they have been gaining interest in the nutraceutical and cosmetic markets (Laranjinha and Cadenas, 1999). Although flavonoids retain the most important structural characteristics to act as an antioxidant, some phenolic compounds, as caffeic acid, can also have such features. Specifically, an *o*-dihidroxy structure, responsible for the electron delocalization of the phenoxyl radical, and a side-chain conjugated with the phenolic ring on one edge and a carbonyl on the other, where both edges allow stabilization of the phenoxyl radical (Laranjinha and Cadenas, 1999) are the important structural characteristics.

Flavonoids are widely found in edible and non-edible plants (Venturelli *et al.*, 2016), and are characterized by having a 2-phenyl-4H-chromen-4-one structure. These phytochemicals are responsibles for organoleptic characteristics of the food they are found in, and the associated health benefits from their consumption (Ferreyra *et al.*, 2012).

The term "phenolic" embraces a wide range of compounds, all aromatic containing hydroxyl substituents. Despite being similar to its parent compound, phenol, most are multiple substituted, being usually polyphenols. The fact that these compounds are very reactive must be taken into account when working with them, mainly due to the ability to take part of intra or inter molecular hydrogen bonds (Mann *et al.*, 1994). Phenolic compounds can be synthetic, like butylated hydroxytoluene (BHT) or obtained from natural products, like vitamin E (α-tocopherol), gallic, protocatechuic, caffeic or rosmarinic acids, amongst others (Brewer, 2011). In addition, the fact that these compounds show reduced to absent adverse effects contributes to their growing interest (Santos *et al.*, 2010; Custódio *et al.*, 2015). Comparing the structures of caffeic acid with quercetin (*Figure 3.1*), can easily be detected the ArC3 moiety of caffeic acid in the quercetin structure, which is according the fact of caffeic acid being the precursor of flavonoids biosynthesis in plants (Laranjinha and Cadenas, 1999).

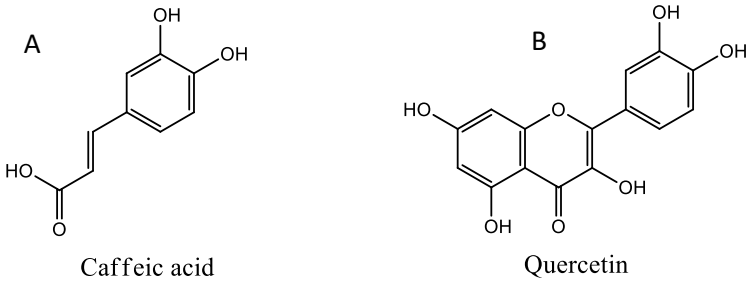

Figure 3.1 - Caffeic acid and quercetin chemical structures [2] A – Phenolic compound; B – Flavonoid.

It is known that botanical extracts have antioxidant activity, specifically, due to the presence of ellagic, gallic, caffeic and protocatechuic acids, vanillin, protocatechuic aldehyde, coniferaldehyde and sinalpehyde (Santos *et al.*, 2010; Brewer, 2011; Custódio *et al.*, 2015).

It becomes of great interest to evaluate antioxidant activity of some natural products, since they have a high content on known antioxidant families, like phenolic compounds or flavonoids (Santos *et al.*, 2013). Since CKSP, evaluated on the present work show a high content on phenolic and flavonoid compounds, several assays were performed aiming to obtain antioxidant activity information towards several ROS/RNS, from different *in vitro* methods (Alam *et al.*, 2013). An example is the DPPH assay that was used to test the capacity of scavenging RNS, where DPPH acts as one, being one of the few commercialized stable nitrogen radicals. The inhibition of lipid peroxidation was also evaluated using linoleic acid as the oxidizable material and AAPH as an oxidizing agent. To test the samples ability to inhibit the activity of peroxyl radical (as an oxidant), ORAC assay was used.

[2] Addapted from Laranjinha and Cadenas, (1999)

3.2 Materials and reagents

3.2.1 Materials

The following reagents were used: (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Polyethylene glycol sorbitan monolaurate (Tween 20), 2,6-Di-tert-butyl-4-methylphenol (BHT) and 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), potassium chloride, potassium dihydrogen phosphate, and disodium fluorescein purchased from Sigma Aldrich (Steinheim; Germany); boric acid purchased from BHD Chemicals, Ltd (Pool, England), linoleic acid 97% from Alfa Aesar (Karlshure, Germany), and sodium hydroxide from V. Reis, Lda (Lisboa, Portugal).

3.2.2. Equipment

The following equipment was used: a *Metrohm 744 pH Meter* (Precision ± 0.01), with a *Metrohm idrolyte* electrode; a *METTLER AE 200* balance (Precision ± 0.0001 g), 96 wells microplates, automatic pipettes. Absorbance measurements were performed using a *SPECTROstar Omega* micro plate reader (Precision ± 0.001 AU), controlled by *Omega* software, and fluorescence measurements were performed using a *Anthos Zenyth 3100* spectrofluorimeter *(Precision* ± 0.001 FU), controlled by *Anthos Zenyth Multimode Detectors* software. For the absorbance measurements, a *Hitachi U-2000* spectrophotometer was used.

3.3 Methods

3.3.1 Samples preparation

3.3.2 DPPH radical-scavenging activity

The free radical scavenging activity of CKSP was determined by the DPPH method, based on the decay of purple colour of DPPH in the presence of antioxidant agents, reported by Sarikurkcu *et al.* (2009).

In this case, DPPH, one of the few stable nitrogen radicals, is used to generate free radicals and test the antioxidant activity of a sample. If hydrogen donor compounds are present on the sample, they will scavenge free radicals (Lewis, 2012), according to *Figure 3.2*. DPPH in its radical form presents a purple colour (in solution) and after being protonated changes to a pale yellow, that will allow a visual confirmation of the reaction previously mentioned. If a variation on the solution colour occurs, it is possible to measure the absorbance to monitor the reaction on a quantitative approach. Usually, wavelengths between 515-528 nm are used, for representing the maximum absorbance of the DPPH radical.

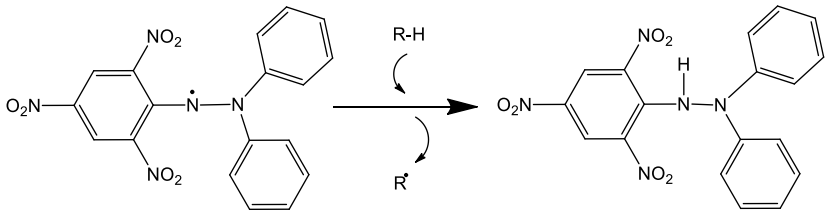

Figure 3.2 - Reaction of DPPH radical with a hydrogen donor.[3]

[3] Addapted from *"Natural Product Screening: Anti-oxidant Screen for Extracts"*, Marsha J. Lewis (2012)

To perform the DPPH assay, samples were processed and analysed. In a 96-well microtiter plate, 180 µL of a DPPH solution (150 µmol L^{-1}) were added to 20 µL of each sample dilution and the plate was shaken for 60 s at 240 rpm min^{-1} (using an *IKA KS 130 basic*). After 40 min of incubation in the dark at room temperature, the absorbance at λ= 517 nm was measured, using a *SPECTROstar Omega* micro plate reader. All solutions were prepared on methanol/water (8:2) and this solution was used as blank. Water was used for the negative control, replacing the sample on the described methodology, and the blank was made with water and 180 µL of the solvent used to prepare the solutions. Trolox solutions with 50, 75, 100, 200 300, 400, 450 and 500 µmol L^{-1} were prepared from a trolox solution (500 µmol L^{-1}) and were used to obtain a calibration curve, allowing to express the results as Trolox Equivalents (TE/ µmol L^{-1}).

The % DPPH quenched was calculated based in *Equation 3.1*:

$$\%DPPH\ quenched = \left(\frac{A_{blanck} - A_{sample}}{A_{blanck}}\right) \times 100 \qquad \textit{Equation 3.1}$$

Where A_{sample} is the absorbance of the sample ; A_{blanck} is the blanck absorbance. All samples and samples dilutions absorbance were also determined in the absence of the radical initiator. Assays were performed in triplicate, and the results were expressed as the mean ± SD.

3.3.3 Inhibition of lipid peroxidation

The ability of CKSP to inhibit lipid peroxidation was determined by the AAPH method, based on the absorbance at λ= 234 nm of the free radicals generated by AAPH, as described by Liégeois *et al.* (2000). After a molecular rearrangement, the lipid radical reacts with the molecular oxygen to form a lipid peroxide (Young and Mceneny, 2001). The last one abstracts a hydrogen from a water molecule, generating another high reactive radical that will start another cycle of peroxidation. This cycle is illustrated in *Figure 3.3*.

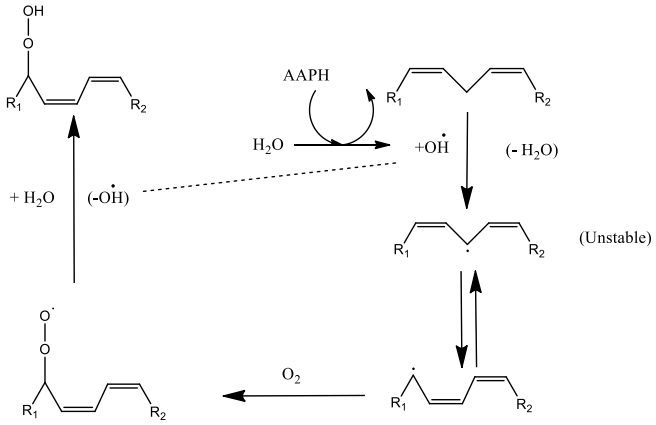

Figure 3.3 - Lipid peroxidation cycle[4].

Since this turns into a cycle, the only way to stop it is by either acting on the high reactive radical species or on the peroxide radicals. Since phenolic compounds are known to be peroxyl radical scavengers (Young and Mceneny, 2001), that will induce the break of the cycle reaction. Due to their hydroxyl groups, phenolic compounds act as a decoy, allowing lipids to

[4] Addapted from "*Lipoprotein oxidation and atherosclerosis", Biochemical Society Transactions* (200I), Volume 29, part 2

escape peroxidation. Azo compounds revealed themselves of great use in lipid peroxidation studies, by generating free radicals through spontaneous decomposition, induced by temperature. Commonly used due to its properties, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) that can be easily manipulated in the laboratory and the water soluble structure allows studies of lipid peroxidation to be performed in an aqueous media.

Beforehand, a linoleic acid dispersion was prepared. Linoleic acid (0.25 mL) was added dropwise to 5 mL of a borate buffer (0.1 mol L^{-1}, pH= 9.00) already containing 0.25 mL of Tween 20. Briefly, 30 µL of a linoleic acid (16 mmol L^{-1}, with Tween 20) dispersion in borate buffer (0.1 mol L^{-1}, pH= 9.0) were added, in a test tube, to 2.8 mL of phosphate buffer (0.1 mol L^{-1}, pH= 7.4) and heated to 37 ºC for 10 min. After adding 20 µL of each sample, 150 µL of an AAPH solution (40 mmol L^{-1}) prepared in phosphate buffer (0.1 mol L^{-1}, pH= 7.4,) were added to initiate the oxidation reaction. After 20 min in the dark and at room temperature, absorbance at 234 nm was measured using a *Hitachi U-2000* spectrophotometer. A BHT solution (90 µmol L^{-1}) was used as a positive control, replacing the sample on the described procedure. A blank solution was obtained with phosphate buffer replacing the sample. Each sample absorbance was also measured.

The percentage of lipid peroxidation inhibition was calculated according to *Equation 3.2*:

$$I(\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \qquad \textit{Equation 3.2}$$

Where A_{blank} is the absorbance of the blank, A_{sample} is the absorbance of the sample. Inhibition of lipid peroxidation assay was performed in triplicates, and the results were expressed as mean ± SD.

3.3.4 Oxygen Radical Absorbance Capacity (ORAC)

Oxygen Radical Absorbance Capacity (ORAC) of samples was obtained by measuring the decay in fluorescence from disodium fluorescein reduced form, induced by the APPH. This method was reported by Huang *et al.* (2002), and measures the ability of a sample to stop disodium fluorescein oxidation process catalysed by peroxyl radicals. This assay is based on the fluorescence loss over time, using fluorescein as the probe, with AAPH generating peroxyl radicals (ROO·) at 37º C. The first step is using the azo initiator (AAPH) to generate radical compounds, according to *Figure 3.4* (Purcell, 2005).

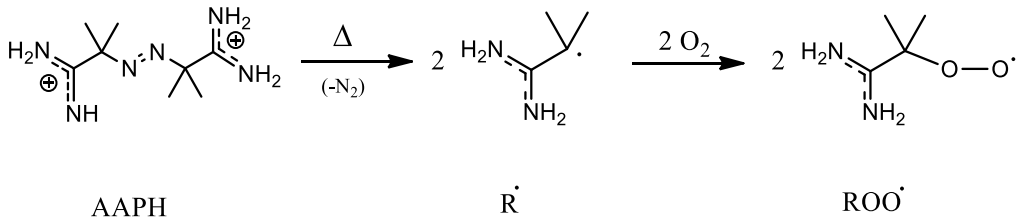

Figure 3.4 - AAPH azo initiator production of ROO· compounds.

After peroxyl radicals are generated, they oxidize fluorescein, leading to fluorescence loss (*Figure 3.5*). This reaction involves several pathways, leading to less fluorescent or non-fluorescent compounds at all (Ou *et al.*, 2001).

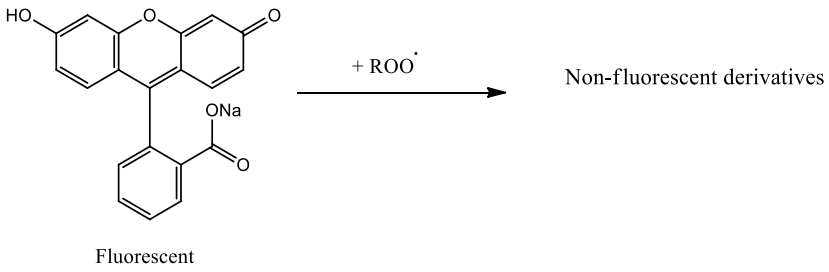

Figure 3.5 - ORAC reaction scheme.

Samples were processed and tested. Phosphate buffer (0.1 mol L^{-1}, pH= 7.4) was used to prepare all solutions, unless it is said otherwise.

Briefly, in a black 96-well MTP, 25 µL of sample were added to 150 µL of a disodium fluorescein solution (4 µmol L^{-1}), and incubated at 37 ºC. After 10 min, 25 µL of an AAPH solution (153 mmol L^{-1}) were added. Fluorescence emitted by the reduced form of disodium fluorescein was measured every 3 min for 2 hours and 20 min, with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Phosphate buffer was used as blank and Trolox solutions (5, 10, 20, 30 40, 80, 100 and 150 µmol L^{-1}) were used as positive controls to generate a calibration curve. Additionally, samples fluorescence was also measured in the absence of disodium fluorescein and APPH.

The final ORAC value for each sample and respective dilutions was calculated on 2 steps. First, using *Equation 3.3* to calculate the area under the curve (AUC), and then using AUC to calculate the Trolox equivalent ORAC value, using a calibration curve with a regression (*Equation 3.4*).

$$AUC = 0{,}5 + \sum_{i=1}^{47} \frac{f_i}{f_1} + 0{,}5{,}\times \frac{f_{48}}{f_1} \qquad \textit{Equation 3.3}$$

$$y = (y_0 - y_{t=\infty}) \times e^{-kx} + y_{t=\infty} \qquad \textit{Equation 3.4}$$

In *Equation 3.3 AUC* is the area under the curve, f_i is the fluorescence when AAPH is added, and f_{48} is the fluorescence of the last measurement.

After plotting the Trolox AUC *vs* Trolox concentration, a non-linear regression (Non-linear – One phase decay) was applied, using *Graphpad*. From the obtained regression, *Equation 3.4*, y_0 is the AUC before the APPC was added, $y_{t=\infty}$ is the y at infinite time and *k* is the rate constant. The obtained results were expressed as Trolox equivalents (TE/µg mL^{-1}).

3.4 Results

3.4.1 DPPH radical-scavenging activity results

The radical-scavenging activity was determined through the DPPH method. CKSP ability to scavenge radicals was assessed and the concentration corresponding to a 50 % reduction in scavenging activity was determined (IC_{50}).

3.4.2 Inhibition of Lipid peroxidation

Lipid peroxidation is an oxidation process that can be initiated with several oxidizing species, like oxygen based free radicals ($O_2^{\bullet -}$, $OH^{\bullet -}$) or with molecular oxygen, allowing oxygen quenchers to stop these reactions, just like phenolic compounds (Liégeois *et al.*, 2000). The lipid gets a hydrogen abstracted by a high reactive radical species (*e.g.* a hydroxyl radical), turning itself into a radical and then passing through several steps until reaching the lipid peroxide.

3.4.3 Oxygen Radical Absorbance Capacity (ORAC)

After peroxyl radicals are generated, they oxidize fluorescein, leading to fluorescence loss. This reaction involves several pathways, leading to less fluorescent or non-fluorescent compounds (Ou *et al.*, 2001).

3.5 Conclusion

The antioxidant properties of CKSP were assessed by studying the sample: i) DPPH radical scavenging activity properties, ii) the ability to inhibit lipid peroxidation and iii) the oxygen radical absorbance capacity.

From the DPPH obtained results, CKSP revealed high AO capacity. The results towards the inhibition of oxygen radicals obtained from ORAC assay point that these kind of samples also have the ability to decrease the oxidation processes from that kind of radicals.

A correlation between the results obtained from *Folin & Ciocalteu* method and ORAC values were already reported in the literature by Casettari *et al.* (2012).

Such a great potential shown by these kind of samples makes them a source of natural AO compounds.

Chapter 4 Antimicrobial activity

"The bamboo that bends is stronger than the oak that resists"

Japanese Proverb

4.1 Introduction

The composition of cork, namely on suberin, lignin, polysaccharides and extractable components (where phenolic compounds are included) varies considerably even for cork-oak trees on the same forest (Fernandes *et al.*, 2011). The phenolic compounds present on cork oak have shown many benefits such as anti-inflammatory, anti-allergic, anti-viral and anti-bacterial activities, and studies focused on its antimicrobial properties have gained little attention (Fernandes *et al.*, 2011; Gonçalves *et al.*, 2016). Several phenolic compounds extracted from plants have been associated to antimicrobial efficiency and among those are gallic acid (Mahadlek *et al.*, 2012), coumarin, curcumin, ellagic acid, epicatechin (Cetin-Karaca and Newman, 2015a), *p*-hydroxybenzoic acid, methyl galate, epicatechin (Gañan *et al.*, 2009), kaempferol and rutin (Pinho *et al.*, 2014).

Over the last few years, several phenolic compounds and flavonoids have been tested for antimicrobial activity. This is related to the urgent need for the discovery of safer drugs not so prone to the occurrence of tolerance/resistance as observed from the misuse of antibiotics (Sohn *et al.*, 2004; Alves *et al.*, 2013). Thus, several plant derived phenolic compounds, also reported to be cork constituents (*e.g.* ellagic acid, caffeic acid and *p*-coumaric acid) (Santos *et al.*, 2010; Touati *et al.*, 2015), have shown planktonic antimicrobial activity towards different bacteria species such as *E. coli*, *Salmonella paratyphi*, *Salmonella chlolerasuis*, *Salmonella Enteritidis* (Cetin-Karaca and Newman, 2015b), *Bacillus subtilis*, *Bacillus cereus*, *Bacillus polyxyma*, *Listeria monocytogenes*, *Clostridium perfringens*, *Clostridium butyricum*, *Clostridium sporogenes* (Cetin-Karaca and Newman, 2015a), methicillin-susceptible (MTS) *Staphylococcus aureus*, methicillin-resistant (MTR) *Staphylococcus aureus* and *S. epidermidis* (Alves *et al.*, 2013). From those studies MIC has been determined either by the disk diffusion or by the microdilution broth and promising results have been obtained mainly with gallic and caffeic acids, which revealed antibacterial activity without damage to animal cells (Pinho *et al.*, 2014).

Besides existing in the planktonic form bacteria can also exist in the sessile stage and form large communities. In the last situation bacteria adhere to a specific surface, grow, form clusters with other similar bacteria and originate biofilms, where they are able to form multiple layers and communicate with each other through the extracellular matrix (ECM) (Satpathy *et al.*, 2016). Recent studies reveal that the communication between biofilm bacteria might create more virulent strains (Satpathy *et al.*, 2016). It is known that the biofilm structure helps bacteria to survive severe environmental conditions and reveals much higher resistance/tolerance to antibiotics when compared to planktonic ones, due to diffusion barriers, mutations concerning a biofilm-specific biocide-resistant bacteria and the biofilm complex matrix. The fact that dead cells also exist inside the biofilm contributes to resistance development, since they act as a dilution gradient for antimicrobial agent *per* cell basis (Skogman, 2012). Hence, it is necessary to develop strategies able to stop the growth of microorganisms as a biofilm structure.

Therefore, since CKSP samples had a high content on phenolic and flavonoid compounds, the aim of this chapter was to test the antimicrobial activity of those samples towards growth inhibition of different microorganisms. The antimicrobial activity was evaluated towards planktonic and sessile cells (biofilm).

4.2 Material and methods

4.2.1 Chemicals

The following chemicals were used: Agar Bacteriological (Nº1) purchased from Oxoid (Hampshire, England); Trypto-casein Soy Broth (TSB), Müller Hinton Agar (MHA), Müller Hinton Broth (MHB) and yeast extract acquired from Biokar (Beauvais, France); sodium chloride, potassium chloride, *n*-hexane and methanol from Merck (Darmstadt, Germany); D-(+)-Glucose monohydrate, resazurin sodium salt, sodium monobasic phosphate, potassium dibasic phosphate, calcium chlorate, magnesium chlorate and Levofloxacin from Sigma-Aldrich (Steinheim; Germany); formic acid from AnalaR (Pool, England); liquid silicone rubber and rubber catalyst from Waker (Munich, Germany); RPMI 1640 from Gibco Life Technologies (Paisley, UK); Brain Heart Infusion (BHI) and peptone from Liofilchem (Bacteriology Products) (Roseto degli Abruzzi, Italy).

4.2.2 Equipment

Media and material were sterilized in a *Mediline Italia* autoclave and a *PBI Miniflo* chamber assured sterile environment when preparing the material for the assays. To arrange the inoculum suspensions, a *Hitachi U-2000* spectrophotometer was used. An *IKA MS3 digital* and an *IKA KS 130 basic* stirrers were also required.

4.2.3. Samples

4.2.3 Microorganisms, culture conditions and sample preparation

Microorganisms, from a stock solution kept at -80 ºC, were seeded in appropriate media (*i.e.* TSA for bacteria and GPYA for yeasts) and used in the following assays.

4.3 Antimicrobial activity

4.3.1 Planktonic cells assays

4.3.1.1 Disk diffusion assay

The disk diffusion assay was used to test the samples capacity to inhibit microorganisms from growing on an agar based medium, according to the Clinical and Laboratory Standards Institute (CLSI) (C.L.S.I., 2012). This assay allows growth inhibition results to be obtained by measuring inhibition zones in agar based medium inoculated with the tested bacteria/yeast.

Inoculum was prepared from 24 h/72 h culture slants of bacteria or yeast respectively, and standardized in appropriate media according to direct colony suspension method. The inoculum turbidity was adjusted to a 0.5 McFarland standard, using a spectrophotometer (*Hitachi U-2000*). MHA plates were inoculated with the different bacteria species, and the antimicrobial disks containing 100 µL of sterile samples were applied. The plates were allowed to incubate at 37 ºC for 18 h. The yeast was inoculated in GPYA and incubated at 37 ºC for 48 h. Inhibition halos were measured with a Vernier caliper.

Levofloxacin aqueous solution (0.5 mg mL^{-1}) was used as the positive control when testing bacteria, and water was used as negative control.

4.3.1.2 Determination of minimum inhibitory dilution

Minimum Inhibitory Dilution (MID) was determined by the Broth Microdilution Method according to CLSI (C.L.S.I., 2012). Successive two fold dilutions of samples were performed in a 96-well MTP with MHB supplemented with Ca^{2+} (15 mg L^{-1}) and Mg^{2+} (4 mg L^{-1}) and RPMI 1640 for bacteria and yeasts, respectively.

Inoculum were prepared by direct suspension from isolated colonies selected from 24 h/72 h cultured agar plate and turbidity was adjusted to a 0.5 McFarland standard. Further dilutions were performed in order to obtain $5x10^5$ CFU mL^{-1} per well when adding 100 µL of inoculum to a complete volume of 200 µL per well.

After 24 h or 48 h (for bacteria and yeast, respectively) of incubation at 37 ºC and visual inspection of the MTP, the MID was determined by measuring the absorbance at λ= 595 nm in an *Anthos Zenyth 3100* Multimode detector, controlled by *Anthos Zenyth Software*. All assays were performed with negative controls (non-inoculated samples and non-inoculated medium) and positive controls (inoculated media).

Additionally, the relative growth (%) compared to positive control wells was calculated using *Equation 4.1*.

$$\textbf{Relative growth}\ (\%) = \frac{\textbf{Abs}_{\textbf{Sample}}}{\textbf{Abs}_{\textbf{PC}}} \times \mathbf{100} \qquad \textit{Equation 4.1}$$

Where Abs_{PC} is the absorbance of the positive control and Abs_{Sample} is the absorbance of the sample. Assays were carried out in 3 independent experiments and results were presented as mean ± SD.

4.3.1.3 Determination of the viability of planktonic microorganisms

Cells viability was determined by the resazurin method. The resazurin method is based on resofurin fluorescent properties. Resazurin (*Figure 4.1*) is a non-fluorescent compound that gets metabolized by living cells into resofurin, a fluorescent one, allowing to assess metabolic activity of cells.

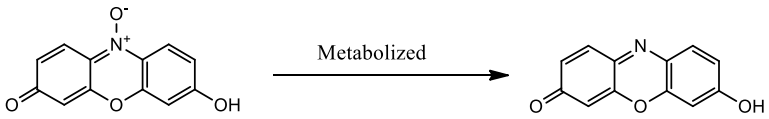

Figure 4.1 – Metabolization of resazurin to resofurin.

After measuring the absorbance in the MID assay, 20 µL of resazurin (1600 µmol L^{-1} prepared by dilution of a 4000 µmol L^{-1}) were added to each well. The MTP was incubated for 20 min at 37 ºC, and fluorescence emitted by the metabolized form of resazurin was measured, in an *Anthos Zenyth 3100* Multimode detector, controlled by *Anthos Zenyth Software*, with 535 nm excitation wavelength and 595 nm emission wavelength.

Relative cellular viability (%) compared to positive control wells was calculated by *Equation 4.2*.

$$\textbf{\textit{Cellular viability}}\ (\%) = \frac{\textbf{\textit{Fluorescence}}_{\textbf{\textit{Sample}}}}{\textbf{\textit{Fluorescence}}_{\textbf{\textit{PC}}}} \times \mathbf{100} \qquad \textit{Equation 4.2}$$

where $Fluorescence_{PC}$ is the fluorescence of the positive control and $Fluorescence_{Sample}$ is the fluorescence of the sample. Assays were carried out in 3 independent experiments and results were presented as mean ± SD.

4.3.2 Biofilm formation inhibition

For the biofilm formation inhibition assays, two different surfaces were tested. Glass and silicone were used to test the capacity of the samples to inhibit cellular growth on hydrophilic and hydrophobic surfaces, respectively. Silicone was prepared by adding 1.2 g of rubber catalyst to 10.8 g of liquid silicone rubber and mixed using a mortar and pestle to help the polymerization reaction. After, the obtained polymer was placed on a mould and left curating for 24 h. The obtained silicone layer was cut into 0.8 cm squares with a thickness of 0.2 cm. Those segments were washed on a beaker for 1 h, under magnetic agitation, changing the water each 30 min. Segments were then dried and sterilized in a *Mediline Italia* autoclave.

Round glass discs with 1.3 cm diameter from Sarstedt were required for this assay. Prior to the surface coating, the disks were sterilized and dried.

Sterile glass discs and silicon segments were fixed inside the wells of a 24-wells MTP. After fixation, 1 mL of sample was added to each well, and MTP were agitated (240 rpm) in an *Ika KS 130 basic*. After 24 h, the samples content were removed from the wells, and 900 µL of BHI (supplemented to 1% (w/v) of Glucose) were added. To prepare the standardized inoculum, turbidity was adjusted to a 0.5 McFarland units and further dilutions were adjusted to obtain $3x10^6$ CFU mL^{-1} per well. From that inoculum, 100 µL were added per well, to a complete volume of 1000 µL. The microplates were then incubated at 37 ºC for 24 h. Culture media was

removed from each well and 1 mL of a phosphate buffer solution (PBS) was used to wash the wells, twice, and each surface biofilm was re-suspended on 1 mL BHI (1% Glucose) (**S**), by scratching and vortexing for 4 min.

4.3.2.1 Biofilm Resazurin assay

From each suspension, two-fold dilutions were made. From those dilutions, 18 µL were added to 162 µL of MHB (1% Glucose), and 20 µL of resazurin (1600 µmol L^{-1}) were used to test the biofilm viability. Fluorescence was measured after 20 min at 37 ºC, using an *Anthos Zenyth 3100* Multimode detector, controlled by *Anthos Zenyth Software*, with a 535 nm excitation wavelength and a 595 nm emission wavelength. Relative biofilm viability (%) compared to positive control wells was calculated by *Equation 4.3*.

$$\textbf{\textit{Biofilm viability}}\ (\%) = \frac{\textbf{\textit{Fluorescence}}_{\textbf{\textit{Sample}}}}{\textbf{\textit{Fluorescence}}_{\textbf{\textit{PC}}}} \times \mathbf{100} \qquad \textit{Equation 4.3}$$

Where $Fluorescence_{PC}$ is the fluorescence of the positive control and $Fuorescence_{Sample}$ is the fluorescence of the sample. Assays were carried out in 3 independent experiments and results were presented as mean ± SD.

4.3.3 Cells hydrophobicity

The hydrophobicity of the biofilm producing bacterial cells was evaluated according to the method proposed by Sodagari *et al.* (2013).

From 24 h slants, a direct colony suspension was prepared in 3 mL of TSB and incubated for 5 h at 37 ºC under agitation (240 rpm) using an *Ika KS 130 basic* stirred. The suspension was vortexed, centrifuged for 5 min (10,000 rpm) using a *Heraeus Biofuge Pico* centrifuge, and supernatant was removed. Cells were washed 3 times with 2 mL of PBS and a final suspension in 3.2 mL of PBS with an optical density of 0.400 (at λ= 600 nm) was prepared. The organic solvent selected for the partition experiment was *n*-hexane and 6 mL of this solvent were added to the 0.400 absorbance suspension. After being vortexed for 2 min, this mixture was left to rest for 30 min. To determine the hydrophobicity, absorbance of the aqueous phase at λ= 600 nm was measured, and partition was calculated, according to *Equation 4.4*.

$$\textbf{\textit{P}}\ (\%) = \textbf{\textit{A}}_{\textbf{\textit{f}}}/\textbf{\textit{A}}_{\textbf{\textit{i}}} \times \mathbf{100} \qquad \textit{Equation 4.4}$$

where *P* stands for the partition, A_i for the initial absorbance, A_f for the final absorbance. The assay was performed in triplicates, and the results were reported as mean ± SD.

4.4 Results

4.4.1 Planktonic cells assays

4.4.1.1 Disk diffusion assay

After incubation, the inoculated petri dishes containing sample impregnated disks were analysed to assure that there were no contaminations and afterwards the inhibition zones diameter were measured.

4.4.1.2 Determination of minimum inhibitory dilution results

4.4.1.2.1 OD600 results

The results obtained through the determination of minimum inhibitory dilution measurement allowed conclusions about the cellular growth on each well, when compared to the initial inoculum absorbance and positive control absorbance.

4.4.1.2.2 Resazurin assay

From the OD600 results, some conclusions could already be taken for the inhibition capacity of the samples. However, that measurement does not differentiate dead cells from the living ones, since it only takes into account the absorbance at λ= 600 nm. In that way, the resazurin assay was used to access viable cells.

From the fluorescence measurements, each well inhibition results was compared to a positive control with the ideal growing conditions, in order to obtain the percentage of viable cells in the tested dilutions.

4.4.2 Biofilm viability results

4.4.2.1 Resazurin assay results

After a coating treatment with CKSP on two distinct type of surfaces, silicon and glass biofilm formation was studied, using resazurin to measure the biofilm viability.

The results obtained for this assay are relative to a coating treatment on the tested surface, to understand if adsorbed compounds present in CKSP can interfere with the adhesion and the biofilm formation of these strains. Like-wise the OD600 and resazurin assays, on this test, each well was compared to a positive control, in order to obtain an inhibition percentage.

4.4.3 Cells hydrophobicity results

Determining the cells hydrophobicity can help understanding the affinity of each used microorganism towards any kind of surface, depending only on its nature.

4.5 Conclusions

With the antimicrobial assays performed to test the properties of CKSP, the profile of this kind of sub-product was studied as a growth inhibitor agent towards several strains of bacteria and yeasts. The tested CKSP revealed growth inhibition capacity.

Chapter 5 Final remarks and future perspectives

“‘Ardet nec consomitur," Melina said. "Burned but not destroyed.”’

Ransom Riggs

From the samples supplied by SOFALCA and studied throughout this work, a new type of CKSP was characterized. Samples presented an acidic profile, with high values of conductivity, turbidity, RI, TDR, TPC and TFC.

Since validation of the method used for quantification of TPC and TFC was mandatory, ISO 8466 (1990) was used as a guideline. Next, compounds known to be cork constituents were identified in the studied CKSP. However, since these compounds were only identified, their quantification can be the aim of a future work. Nevertheless, some non-identified compounds were present on the samples. So, in order to try to fully characterise these CKSP, the remaining un-identified compounds should be studied using LC-MS/MS on negative and positive mode, alongside other characterization techniques, like NMR with fractions of these samples.

Afterwards, both samples antioxidant activity was tested using *in vitro* methods. To assess antioxidant capacity profile towards ROS (peroxide radicals) and RNS (simulated by DPPH free radical), different methods were used. Either towards RNS or lipid peroxidation, CKSP revealed a high scavenge capacity. With such capacity, and knowing the present compounds, this kind of samples could become a source of natural antioxidants. In the future, other antioxidant assays could be used, in order to understand a more suitable application on the industrial field. Peroxynitrite radical scavenging activity assay for peroxynitrite radicals, a stable radical that lacks specific endogenous scavengers and HORAC method for hydroxyl radicals generated by *Fenton*-like reaction as *in vitro* methods are just two examples of methods that could be used.

In terms of antimicrobial activity, several species of microorganisms were tested. Promising results were obtained from the growth inhibition assays and the biofilm viability reduction. Therefore this type of samples showed a potential use as a natural source of active antimicrobial compounds. Due to the inhibition capacity of the studied CKSP, other bacterial species could be tested in the future.

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Annex

iMed.ULisboa Postgraduate Students Commission

Implementation of microscale methods for quantification of phenolic and flavonoid compounds on natural products

Chemical Biology and Toxicology (CBT)

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Phenolic compounds and flavonoids are important secondary natural metabolites, which possess an array of health-promoting benefits and have engaged a great deal of scientific interest due to their health promoting effects as antioxidants. These natural compounds are ubiquitous in plants and they can act as chelators and free radical scavengers.

This study aimed the implementation and validation of micromethods for quantification of phenolic and flavonoid compounds present in natural products. Thus, Folin & Ciocalteu and Aluminium Chloride methods were selected to quantify phenolic and flavonoid compounds, respectively. Both methods had to be adjusted to the microscale approach, and therefore validation was necessary. Validation was performed according to ISO 8466-1 1990, and was focused on the linearity of the method within the working range, Limit of Detection (LOD), Limit of Quantification (LOQ) and the homogeneity of variances.

Folin & Ciocalteu method linearity was accessed (R^2= 0.9992), all normalized residues were within a 15% deviation range and in addition linearity was also confirmed by the *F*-test for linearity (p=0,005). Working range suitability (2-10 ppm) was checked through the homogeneity of variances (p=0.005) and a LOD of 0.27 ppm and LOQ of 0.82 ppm were obtained. In aluminium chloride method the same validation procedures were followed. Linearity was accessed (R^2=0.9955) confirmed through normalized residues and the *F*-test. LOD and LOQ were 1.30 ppm and 3.93 ppm, respectively, and the working range (4-20 ppm) was also accessed (p=0.005).

Both micro methods implemented revealed to be suitable for the assessment of natural products samples content on phenolic and flavonoids compounds.

Acknowledgements: We thank the Fundação para a Ciência e Tecnolgia for financial support of iMed.ULisboa project (UID/DTP/04138/2013).

*Annex I – Gomes, R., Santos, S., Ribeiro, I.A.C., "**Implementation of microscale methods for quantification of phenolic and flavonoid compounds on natural products**", Faculdade de Farmácia da Universidade de Lisboa, 2016*